**Supplementary Materials**

**Supplementary Results**

*Arousal and anxiety*

Movement speed is routinely used as a measure of excitability or arousal, a variable that could influence learning on day one (Archer 1973). Average speed of movements made during forays was comparable for the SH (12.0 ± 0.3 cm/sec) and EE (11.7 ± 0.2 cm/sec) rats during the 30 minute session. Surprisingly, movement speed in the WR rats (10.3 ± 0.2 cm/sec) was less than in the other groups ($F(2,86)=13.55$, ANOVA: $P<0.0001$; SH and EE vs. WR: $P<0.001$; SH vs. EE: n.s., Tukey HSD) (Fig S1A). Percent time moving during an exploratory episode provides a second measure of arousal. Within group variability was low for this index (coefficients of variation were < 15%) and mean values for day one were comparable between the SH (69.5 ± 1.2% time moving) and EE (65.7 ± 1.7%) rats. The WR group (62.9 ±1.6%) was slightly reduced from the SH controls ($F(2,86)=4.66$, ANOVA, $P=0.012$; SH vs. WR: $P<0.01$; other comparisons were not significant) (Fig S1B).

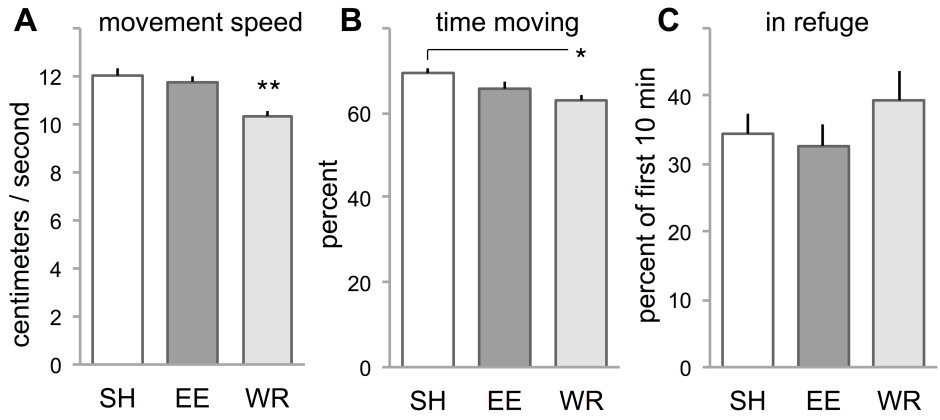


Fig. S1. Little to no evident difference in measures of arousal and anxiety. (A) Speed of movements in the arena during the first session. Unexpectedly, WR group rats showed slightly lower average movement speeds than the other groups. SH and EE rats were not different. (B) Another measure of arousal, percent time moving while in the arena, was similar between the groups, with WR rats slightly lower than SH. (C) Percent of the first 10 minutes of the session that was spent in the refuge; groups did not differ significantly on this measure. (**) $P < 0.001$, (*) $P < 0.01$.

Besides allowing rats to partition their behavior into voluntary forays, a spontaneously emergent behavior, the inclusion of a darkened refuge provides a conventional measure of anxiety in rodents: the tendency to avoid open illuminated spaces. There was no evident difference in this index, as measured by time spent within the refuge, between the SH and EE groups during the first ten minutes following introduction into the apparatus: SH: 28.1 ± 2.3% of time; EE: 31.9 ± 2.8% (Fig S1C); WR values were higher (WR: 37.8 ± 4.4%) but this did not reach significance ($F(2,86)=0.91$, ANOVA: $P=0.41$). Additionally, as noted the (empty, refuge-less) open field test was originally introduced by Hall to

measure anxiety, primarily via quantification of defecations which, in rat, also serves as a conventional measure of anxiety or fearfulness (Hall and Ballachey 1932); in our studies, likely in part due to the presence of the refuge (as well as extensive interactions with experimental personnel during pretreatment for animals in all groups), these counts were very low: usually none, with no group differences (Table S1).

| | Total | 0 | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|---|---|
| **SH** | n=31 | 25 | 3 | 2 | 0 | 0 | 1 | 0 |
| **EE** | n=31 | 27 | 1 | 1 | 1 | 0 | 0 | 1 |
| **WR** | n=27 | 22 | 2 | 0 | 0 | 1 | 1 | 1 |

Table S1. Exposure to the novel test apparatus was not associated with a conventional indicator of anxiety for almost all rats tested and no group differences were observed. Frequency table shows number of rats in each group that left between 0 and 6 droppings during the first session (none left more than 6). Three animals in each group left more than a single dropping, with the large majority leaving none.

These results suggest that past experience had little influence on anxiety and arousal in the novel situation, and indicate that differences in arousal and anxiety were not likely to have contributed to the learning differences between EE rats and the other groups. There was evidence for small effects of prior exercise on movement speed and time moving in WR rats, though no evidence of any difference in anxiety.

*Prior experience and synapse numbers*

Numerous studies have shown that exposure to enriched environments can result in an increase in the number of dendritic branches and spines in rodents (Globus et al. 1973; Greenough et al. 1973; Kolb and Whishaw 1998; Lauterborn et al. 2015) along with improved performance in a variety of rewarded learning tasks (Leggio et al. 2005; van Praag et al. 2000), although exposures are much longer than those used in the present studies. Such effects if translated into greater connectivity between hippocampal neurons would provide a potential explanation for the potent effects of enrichment on learning observed here. We explored this idea by measuring the number of synapses in different hippocampal subdivisions.

A separate cohort of rats given SH and EE pretreatments (n=6 per group) was prepared for synapse counts using immunofluorescence localization of the post synaptic density scaffold protein PSD-95, a protein that is uniformly distributed across post-synaptic densities at excitatory (glutamatergic) contacts (Petersen et al. 2003; Sassoé-Pognetto et al. 2003), and three-dimensional (3-D) reconstructions of labeled puncta using automated counting and measurement systems (see Supplemental Methods, below). EE rats were given one 30 min session in the test apparatus whereas SH rats remained in transport bins for 30 min, then immediately euthanized for tissue collection; in this context the 30 min exploration session was regarded as an enrichment experience and thus not given to the SH group.

Tissue sections were processed for immunofluorescence localization of PSD-95 (Fig S2A). Digital image z-stacks were collected through a depth of 3 µm (0.2 µm steps) from 9 dendritic sample zones from a cross section of rostral hippocampus (Fig S3B) and numbers of synapse sized PSD-95 aggregates were quantified from 3-D reconstructions of each stack.

Mean counts of synapses for the hippocampus (all fields) were not greater in the EE group (SH: 41.6 ± 1.2 psds/105x136x3 µm; EE: 39.5 ± 1.0; $t(18)=1.31$, $P=0.20$). This also held for comparisons of each individual subfield (Fig S3C; $F(1,89)=2.17$, $P=0.14$ 2-way ANOVA, interaction $F(8,89)=1.24$, $P=0.28$).

Synapse size is another variable that relates to communication within hippocampal networks because it correlates with the number of AMPA-type glutamate receptors attached to them (Ottersen et al. 1999); the size of the AMPA receptor pool dictates the size of the EPSC elicited by a release event and thus relates to the potency of individual contacts. The 3-D reconstruction technology enabled estimates of the volume of the tens of thousands of PSD-95 immunolabeled synapses within each sampling field. The frequency distribution for sizes (percent of all contacts that fall into graduated size bins) was described by

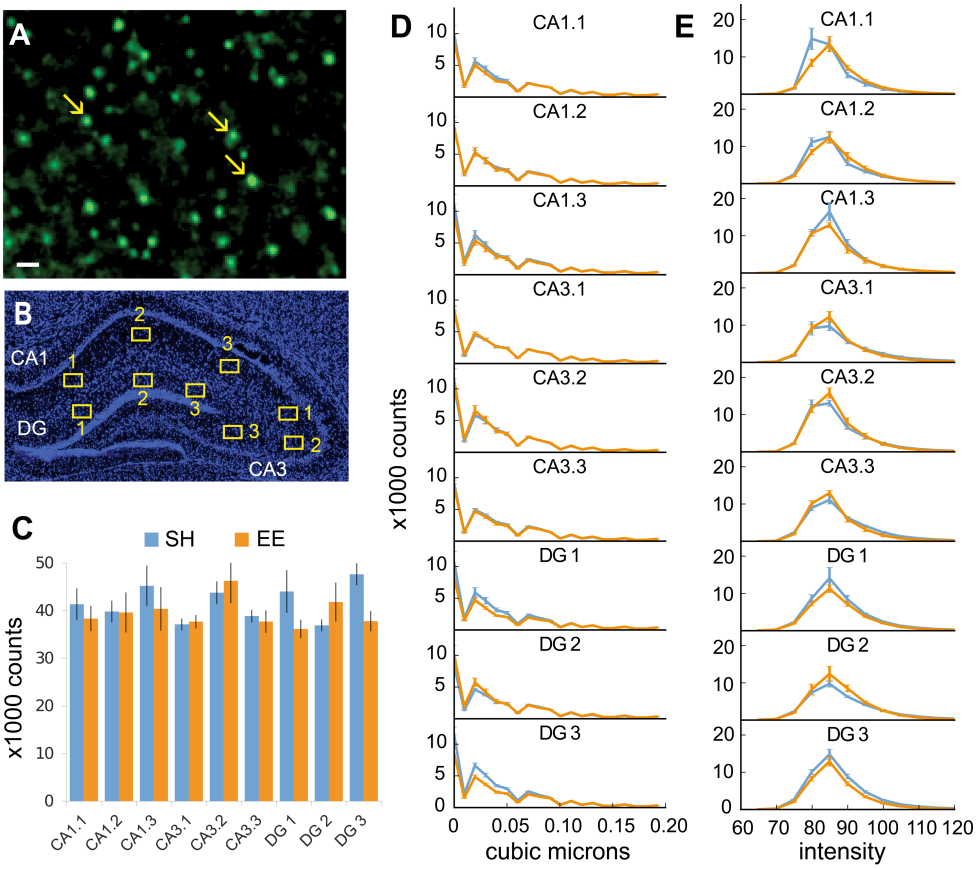


Fig. S2. Synapse counts are not different between groups. (A) Representative image shows punctate PSD-95 immunoreactivity within CA1 sr; arrows indicate example puncta. Scale bar = 1µm. (B) Illustration of sampling zones in each of dendritic fields of CA1, CA3, and dentate gyrus. (C) Mean synapse counts for each subfield sampled were not different between the EE and SH groups. (D) Frequency distribution (percent of all synaptic puncta falling into size bins) for each of the nine subfields. There were no differences in size measurements between the two groups. (E) Frequency distributions for labeling density for each subfield. There were no differences between the two groups.

a Poisson curve, as described for EM measurements (Harris et al. 1992), and was not detectably different between EE and SH brains (Fig S3D, F(1,89)=2.06, P=0.15 on medians, 2-way ANOVA) for overall measures (all fields together) or for each of the individual subfields measured (Fig S3D, F(8,89)=1.31, interaction P=0.25). Next, we measured the density of PSD-95 immunolabeling for individual synapse sized clusters. There were no evident differences between SH and EE animals in the overall summary of regions on this measure (F(1,89)=1.19, P=0.28, 2-way ANOVA) or in comparisons of the nine subfields (Fig S3E, F(8,89)=0.62, interaction P=0.76).

We conclude from these results that six days of experience with a very complex enriched environment does not produce lasting changes to the numbers, size, or structure of the postsynaptic face of hippocampal synapses.

*Replication of learning results for rats used for mapping of a synaptic marker of LTP*

Rats given EE or SH pretreatment were sacrificed at the conclusion of a single 30-minute session in the apparatus, and brain tissue sections collected for mapping of a synaptic marker of LTP (pCofilin), as described in the manuscript. During the session the learning differences (habituation curves) between EE and SH rats on session 1 described in the manuscript (Fig 2) were replicated in these new groups of rats (Fig S3): the slope of this EE group habituation curve was again much steeper than that of these SH rats (Fig S3C: EE vs SH: $t(20.9) = -4.9$, $P < 0.0001$), and the curves for each group (Fig S3B) were very similar to those seen for session 1 of the large cohort described in the manuscript (Fig 2C). However in this much smaller cohort of animals (SH n=12, EE n=11), the total distance traveled during the session (Fig S3A) was not significantly different between the groups.

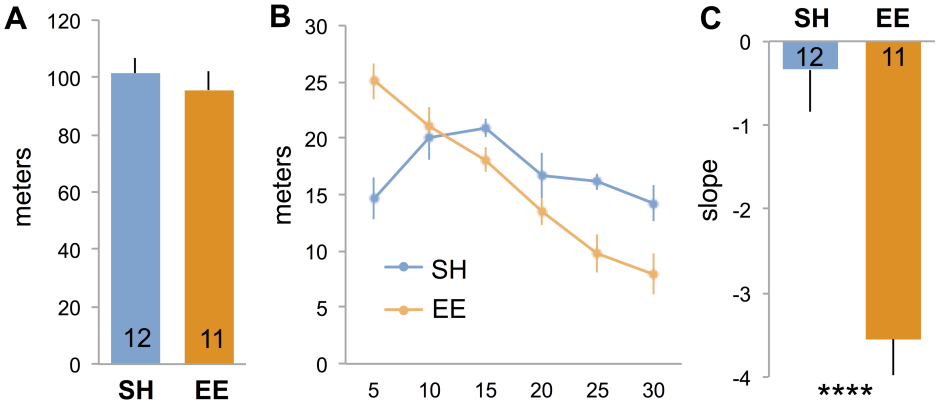


Fig. S3. Enrichment accelerated habituation in the complex arena in a separate cohort of rats euthanized after the first session to collect brain tissue for synaptic measurements (EE n=11, SH n=12). (A) There was no evident difference between SH and EE rats in total distance traveled in the complex arena in this smaller group of animals. (B) Plots of distance traveled in 5 min time intervals in the arena show habituation curves that are very similar to those obtained from the large, behavior-only cohorts described in the manuscript. Here again the EE rats show good habituation within the session while the SH rats do not. (C) Slopes of the curves from panel B are considerably steeper for EE rats than SH rats. (****) $P < 0.00001$.

*Habituation in the complex arena - refuge apparatus as a measure of learning*

Past studies showed that exploration characteristics in rodents are sensitive to arousal levels and psychological state (e.g., anxiety) (Berlyne et al. 1966; Lester and David 1968; Russell 1973); we identified a pair of variables (speed and amount of movement) that was slightly reduced by past exercise. The similarities between the enriched and standard handled rats suggests that state changes in brain were not responsible for the marked differences between these two groups in learning during their initial encounter with the test apparatus. At the same time, we think it would be difficult to establish the direction of causality between any potential physiological or hormonal differences between the groups and the degree to which they reduced their exploration because of learning: either of the two variables could drive the other (familiarization through learning could alter stress or changes in the latter could enable the former). This is of course a problem faced by learning studies in general when dealing with experimental manipulations. In the present case, we did not detect any group effects with regard to multiple behavioral measures conventionally used as indices of fear or arousal. As to whether the rapid decrease in exploration by the EE animals during the initial session was due to learning as opposed to other factors, we note that the behavior of this group was markedly different on the second test day and in the direction predicted by the encoding of memory on day one. This was not the case for the prior exercise or handling cases.

While widely used, the open field paradigm has been the object of several recent and historical critiques (Spruijt et al. 2014; Rodgers 2007; Stanford 2007; Walsh and Cummins 1976; Archer 1973) Many of these focus on its role as a test of anxiety (Prut and Belzung 2003 for review), or how to interpret the meaning of locomotory activity within an aversive open field context: is it an escape reaction or exploratory behavior? For example, in a critique of its use as a "simple behavioral test" of "emotionality" Rodgers (2007) notes that "patterns of ambulation and exploration will be strongly influenced by emotional reactivity and not always in predictable ways", an issue (as he notes) already raised by Walsh & Cummins (1976). In the present studies, we focus on locomotion as a measure of exploratory activity in our complex open field, although there may certainly be elements therein of search for escape routes or for the other rats from which they have been separated for the first time. But we argue that the evident readiness of the animals in all three groups to leave the ethologically 'safer' refuge (Whishaw et al. 2006), along with the lack of other markers of marked anxiety such as defecatory pellets, supports interpretation of the locomotory activity in our study as primarily exploratory. Along these lines, a nicely designed 2000 study compared the effects of three types of open fields on conventionally housed rats: a traditional open field (empty); the same with an attached refuge; and a complex open field (consisting of several chambers with internal complexity including shallow water pools) with an attached refuge. These authors found that the addition of the refuge to the plain open field reduced "emotional responses" (defecation and urination) but rats were disinclined to leave the refuge to enter it (showing

delayed and reduced entrances), while rats readily left the refuge to traverse the complex arena, which the authors considered evidence of genuine exploratory activity (Genaro and Schmidek 2000).

**Supplementary Methods**

Rats were euthanized by decapitation under isoflurane inhalation anesthesia. Brains were fast frozen and cryostat sectioned (20 µm thick, coronal). Series of sections at 200 µm intervals were slide mounted, fixed in cold methanol and processed for immunofluorescence localization of the post synaptic density scaffold protein PSD-95 as described (Seese et al. 2013) using a primary antisera cocktail of mouse anti-PSD-95 (1:1000; ThermoFisher Scientific #MA1-045) and secondary antisera including AlexaFluor488 anti-mouse IgG.

To assess synapse counts, nine digital image z-stacks per hippocampal section were captured through a depth of 3 µm at 0.02 µm steps from sections through rostral hippocampus (-3.24mm from Bregma) using a Leica DM6000 B microscope with a 63 Plan Apo objective. Three-dimensional (3-D) reconstructions of each 135 X 105 X 3 µm target field were generated and qualitatively analyzed for synapse counts, using Volocity V4 (Perkin Elmer) and in house software as described previously (Seese et al. 2013). Our computerized measurement system 3-D reconstructs and counts only those clusters of PSD-95 labeling that satisfy the size and shape constraints of synapses.

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